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(54) **Compositions comprising low molecular weight Heparin**

Zusammensetzungen enthaltend Heparin mit niedrigem Molekulargewicht

Compositions contenant de l'héparin à bas poids moléculaire

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WO-A-98/55515

US-A- 4 981 955

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- **CHOAY, J. ET AL.: "Structural Studies on a Biologically Active Hexasaccharides obtained from Heparin" ANNALS NEW YORK ACADEMY OF SCIENCES, vol. 370, 1981, pages 644-649, XP000918149**
- **DATABASE WPI Week 199408 Derwent Publications Ltd., London, GB; AN 1994062063 XP002146575 "New Tetrasaccharides: Useful as Anticoagulants" (SEIKAGAKU KOGYO CO LTD.), 3 July 1992 (1992-07-03) & PATENT ABSTRACTS OF JAPAN vol. 018, no. 222, 21 April 1994 (1994-04-21) & JP 06 016687 A (SEIKAGAKU KOGYO CO LTD), 25 January 1994 (1994-01-25)**

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Description

[0001] This invention relates to new compositions of heparins of low molecular weight (LMWH) comprising fragments or oligosaccharides.

[0002] Heparin is a mucopolysaccharide sulphate of animal origin, extracted from mammal intestine or lung (cow, lamb, pig) and used for some time now in human therapy for prevention and treatment of thromboembolic diseases. It is well known that the use of heparin is accompanied by very upsetting haemorrhaging effects and its daily administration, three subcutaneous or intravenous injections, constitutes a very considerable drawback.

[0003] During the course of the last few years different chemical methods have been used for depolymerising heparin, such as:

- treatment with sodium nitrite in acid medium
- alkali treatment of esters
- use of free radicals generated in the presence of hydrogen peroxide
- treatment of a quaternary ammonium salt of heparin in non-aqueous medium with a strong base according to a beta-elimination mechanism.

These methods allow variable yields to be obtained, mixtures of fragments of heparin in which the average molecular weight and the anti-coagulant activity vary according to the procedure and the conditions of the operation. The beparins of low molecular weight (LMWH) described in the state of the art or commercialised are obtained according to different depolymerisation procedures. Their average molecular weight (M_w) lies between 4,000 and 6,000 Daltons.

[0004] Currently it is recognised that the anti-thrombotic activity of LMWHs is due principally to their capacity for activating anti-thrombin III, a plasma protein and potent inhibition of activated factor X and thrombin. In this way it is possible to measure the anti-thrombotic activity of heparin by means of tests specific to the inhibition of these factors.

[0005] The research carried out by different authors in recent years shows that fragments or oligosaccharides of heparin consisting of short chains of medium molecular weight < 4,800 Daltons have a selective action on activated factor X and have little effect on the global coagulation measured using methods of the Pharmacopoeia.

[0006] Document 370 644 (Choay et al. Ann. N.Y. Acad. Sci., 1981), describes the chemical structure of a hexasaccharide and an octasaccharide, with proposed structures. The products according to that document have an anti-Xa activity between 2100 and 2400 U/mg.

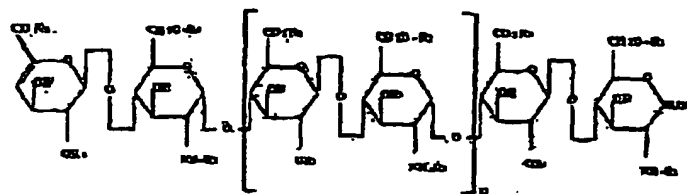
[0007] Documents US-A-4 981 955 and US-A-5 576 30 are related to a process for preparing low molecular heparin derivatives, with an average molecular weight between 4000 and 6000 Dalton.

[0008] Document WO 98/55515 discloses modified low molecular weight heparin derivatives; their average molecular weight is between 5000 and 9000 Dalton, and their anti-factor IIa activity is between 40 and 100 U/mg.

[0009] Document EP-A-0 293 539 refers to a anti-trombic composition comprising a heparin of low molecular weight and dermatan sulphate. Their average molecular weight is approximately 5000 Dalton.

[0010] It has been found that if fragments of very low molecular weight are required that have a strong anti factor Xa activity it is preferable to use a selective depolymerisation technique in non-aqueous medium, as is described in the patent USP 9,981,955 that does not run the risk of attacking the site of binding to anti-thrombin III.

[0011] In view of the background and state of the art described above, the present application has developed, using a procedure in non-aqueous medium, the controlled depolymerisation of heparin that allows a new family of LMWH to be obtained rich in oligosaccharides of low molecular weight that have a high anti-factor Xa activity and a low anti factor IIa activity, and that can be represented by the general formula:



[0012] In which:

n can vary between 1 and 12

$$R_1 = \text{H or SO}_3\text{Na}$$
$$R_2 = \text{SO}_3\text{Na or COCH}_3$$

[0013] Said heparin of very low molecular weight is obtained by selectively depolymerising the heparin in non-aqueous medium according to a procedure of beta elimination.

[0014] The compositions of heparins of very low molecular weight according to this invention are characterised by a molecular weight lying between 2,000 and 4,000 Daltons, an anti factor Xa activity of at least equal to 100 I.U./mg, preferably between 100 and 150 IU/mg and because they contain a strong proportion, up to 75 %, of oligosaccharides of low degree of polymerisation that go from hexasaccharide (n=1) to dodecasaccharide (n=4). These compositions are useful in the prophylaxis and treatment of venous and arterial thrombosis. They can be used as anti-thrombotic medication.

[0015] The LMWHs known and exploited commercially contain small proportions of oligosaccharides of low molecular mass, notably the oligosaccharides whose degree of polymerisation goes from hexasaccharide to dodecasaccharide.

[0016] The compositions of heparin, fruit of the present invention, have as a main characteristic, the fact that they contain a strong proportion, up to 75 %, of such oligosaccharides. Furthermore, these oligosaccharides have a high anti factor Xa activity (> 100 I.U./mg) giving them a long lasting and high anti-thrombotic activity.

Said compositions of heparin have an anti-factor Xa activity lying between 100 and 150 LU/mg and whose anti-factor IIa activity is less than or equal to 10 I.U./mg.

[0017] The average molecular weight of the compositions of heparin of the present application lie between 2,000 and 4,000 Daltons and because:

- they contain from 25 to 60 % of oligosaccharides of molecular weight less than 2,000 Daltons;
- they contain from 40 to 75 % of oligosaccharides of molecular weight lying between 2,000 and 6,000 Daltons; and
- they contain less than 15 % of oligosaccharides of molecular weight greater than 6,000 Daltons.

[0018] The heparin compositions of the present invention are composed of mixtures of oligosaccharides or fragments of heparin. The percentage of fragments that form part of the present invention are as follows:

- contain less than 10 % of fragments in which n lies between 10 and 12;
- contain from 80 to 90 % of fragments in which n lies between 1 and 6; and
- contain less than 15 % of the fragments in which n lies between 7 and 9.

[0019] The present invention is illustrated by the following examples, without these examples limiting the scope of the invention.

[0020] The molecular weight (Mw), the molecular distribution, as well as the anti-factor Xa and anti-factor IIa activities have been determined according to techniques described in the monograph no. 828 "Heparin of low molecular weight" of the Third Edition of the European Pharmacopoeia.

EXAMPLE 1:

[0021] After dissolving 1 kg of unfractionated sodium heparin in 7 litres of purified water, 4.4 litres of a solution of benzalconium chloride in water at 50% w/v is added to the heparin solution while stirring constantly.

Water is added to make up a volume of 30 litres and allowed to decant.

Next the supernatant is removed and water added to make up 30 litres and left to decant.

Once decanted the supernatant is removed and the precipitate liophilised.

Approximately 2.7 kg of benzalconium salt of heparin is obtained (Product A).

After dissolving 100 g of product A in 300 ml of dichloromethane, Triton B® is added in three lots:

- 25 ml of Triton B® is added and the mixture left for 8 hours at 25° C
- 25 ml of Triton B® is added and the mixture left for 16 hours at 25° C
- 25 ml of Triton B® is added and the mixture left for 8 hours at 25° C

The above solution is precipitated over 600 ml of solution of sodium acetate in methanol at 10 % w/v and the precipitate collected by centrifugation with washing with methanol.

[0022] The product obtained is dissolved in 500 ml of water, neutralised with 0.1N HCl, and sodium chloride added until a concentration of 10% w/v is reached. Precipitation is effected by the addition of 1.25 litres of methanol.

Next the precipitate is collected by filtration washing with methanol and drying under vacuum at 35° C to give 33 g of Product B, which is dissolved in water at 10% w/v.

The temperature is brought to 25° C and sodium chloride added to give a concentration of 10 % w/v. Precipitation is effected by adding 2.5 volumes of methanol. The precipitate is then collected by filtration washing with methanol and drying under vacuum at 35° C obtaining 26 g of purified product, which is dissolved in water at 5 % w/v.

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The pH is adjusted to 6.6 using 0.1 N HCl and sodium chloride added to give a concentration of 5 % w/v.

Precipitation is effected by addition of 0.8 volumes of methanol.

After collecting the precipitate by filtration washing with methanol, the product is dried under vacuum at 35° C.

The supernatant is precipitated with 1.6 volumes of methanol.

5 This precipitate is collected by filtration washing with methanol and drying under vacuum at 35° C.

At the end of the process 22 g of product is obtained.

EXAMPLE 2:

10 **[0023]** The method for obtaining Product B in example 1 is repeated.

20 g of product B are dissolved in 150 ml of water and 100 ml of a solution of benzalconium chloride in water at 50 % w/v added.

Next, water is added to make the mixture up to a volume of 500 ml. The mixture is left to decant.

Once decanted the supernatant is removed, water added to make up a volume of 500 ml and the mixture left to decant.

15 After withdrawing the supernatant the precipitate is lyophilised to give 50 g of benzalconium salt.

20 g of the salt obtained are dissolved in 60 ml of dichloromethane.

5 ml of Triton B® are added and the mixture left for 8 hours at 35° C.

Next, the above solution is precipitated over 120 ml of a solution of sodium acetate in methanol at 10 % w/v and the precipitate collected by centrifugation washing with methanol.

20 The product obtained is dissolved in 100 ml of water, neutralised with 0.1N HCl, and sodium chloride added until a concentration of 10% w/v is reached. Precipitation is effected by addition of 250 ml of methanol.

The precipitate is collected by filtration, washing with methanol and drying under vacuum at 35° C.

At the end of the process 6.3 g of Product is obtained.

25 **EXAMPLE 3:**

[0024] Initially 5 g of the product obtained in Example 2 are dissolved in water at a concentration of 5 % w/v.

The pH is adjusted to 6.6 with 0.1 N HCl and sodium chloride added until a concentration of 5% w/v is reached.

Precipitation is effected by addition of 1.5 volumes of methanol.

30 Next, the precipitate is collected by filtration washing with methanol and drying under vacuum at 35° C.

At the end of the process 3 g of Product is obtained.

EXAMPLE 4:

35 **[0025]** The method for obtaining product B described in Example 1 is repeated.

20 g of product B are dissolved in 150 ml of water and 100 ml of solution of benzalconium chloride in water at 50 % w/v added.

Water is then added to make up a volume of approximately 500 ml.

After withdrawing the supernatant, water is added to make up a volume of 500 ml, and the mixture left to decant.

40 The supernatant is withdrawn, water is added to make up a volume of 500 ml, and the mixture left to decant.

After dissolving 20 g of the salt obtained above in 60 ml of dichloromethane, Triton B® is added in two lots:

- 5 ml of Triton B® is added and left standing for 8 hours at 35° C
- 5 ml of Triton B® is added and left standing for 16 hours at 35° C

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[0026] The above solution is precipitated over 120 ml of solution of sodium acetate in methanol at 10 % w/v and the precipitate collected by centrifugation washing with methanol.

The product obtained is dissolved in 100 ml of water, neutralised with 0.1N HCl, and sodium chloride added until a concentration of 10 % w/v is reached and precipitation effected by addition of 250 ml of methanol.

50 The precipitate is then collected by filtration washing with methanol and drying under vacuum at 35° C.

At the end of the process 8.7 g of product is obtained.

EXAMPLE 5:

55 **[0027]** Initially, 5 g of the product obtained in example 4 are dissolved in water at 5% w/v.

The pH is adjusted to 6.6 with 0.1N HCl and sodium chloride added until a concentration of 5% w/v is reached.

Next, precipitation is effected by addition of 0.93 volumes of methanol.

After collecting the precipitate by filtration and washing with methanol, the product is dried under vacuum at 35° C.

[0028] The supernatant is precipitated with 2 volumes of methanol. The precipitate is collected by filtration washing with methanol and drying under vacuum at 35° C. Finally, 4 g of product are obtained.

5 ANALYSIS OF THE PRODUCTS

[0029]

	Anti-coagulant activity (I.U./mg)	
	Anti Xa	Anti IIa
Product of Example no. 1	120	8
Product of Example no. 2	105	3
Product of Example no. 3	110	6
Product of Example no. 4	100	4
Product of Example no. 5	100	2

	Molecular weight Mw	Molecular distribution		
		*	**	***
		< 2,000	2,000 - 6,000	> 6,000
Product of Example no. 1	3,650	25	70	5
Product of Example no. 2	3,195	35	55	11
Product of Example no. 3	3,616	24	63	13
Product of Example no. 4	2,777	42	52	7
Product of Example no. 5	2,161	57	41	2

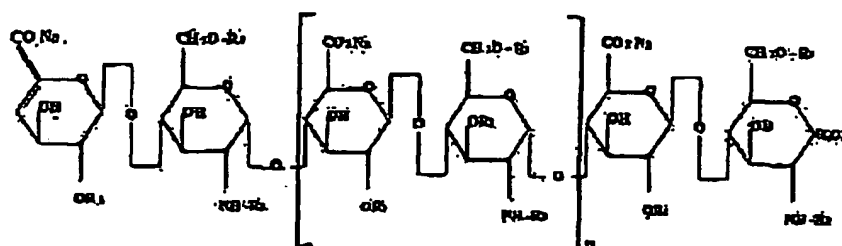
* % of oligosaccharides of molecular mass less than 2,000 Daltons

** % of oligosaccharides of molecular mass lying between 2,000 and 6,000 Daltons

*** % of oligosaccharides of molecular mass greater than 6,000 Daltons

Claims

1. A composition of heparins having a very low molecular weight and comprising fragments or oligosaccharides of general formula:



wherein:

n can vary between 1 and 12

R₁ is H or SO₃Na and

R₂ is SO₃Na or COCH₃,

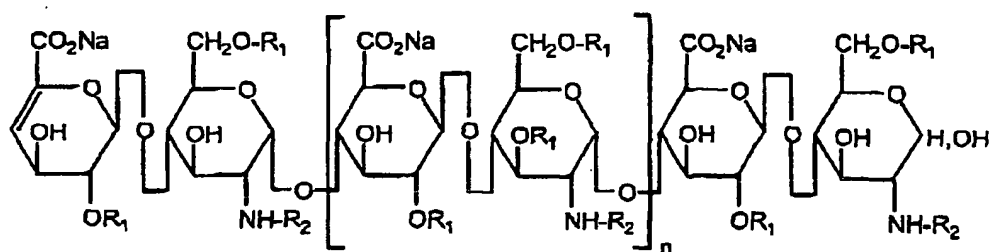
characterized in that said composition comprises less than 10 % of fragments or oligosaccharides of said

general formula wherein n lies between 10 and 12; from 80 to 90 % of fragments or oligosaccharides of said general formula wherein n lies between 1 and 6; and less than 15 % of fragments or oligosaccharides of said general formula wherein n lies between 7 and 9; and in that from 25 to 60 % of said oligosaccharides have a molecular weight less than 2000 Daltons, from 40 to 75 % of said oligosaccharides have a molecular weight lying between 2000 and 6000 Daltons and less than 15 % of said oligosaccharides have a molecular weight greater than 6000 Daltons, the average molecular weight (Mw) of the composition lying between 2000 and 4000 Daltons; whereby said composition exhibits a high anti-factor Xa activity between 100 and 150 I.U./mg and a low anti-factor IIa activity less than or equal to 10 I.U./mg.

2. Use of the composition according to claim 1 in the preparation of an anti-thrombotic medicament.

Patentansprüche

1. Zusammensetzung aus Heparinen, die ein niedriges Molekulargewicht aufweisen und Oligosaccharidfragmente aus Oligosacchariden der allgemeinen Formel



in welcher

n zwischen 1 und 12 variieren kann,

R_1 H oder SO_3Na ist und

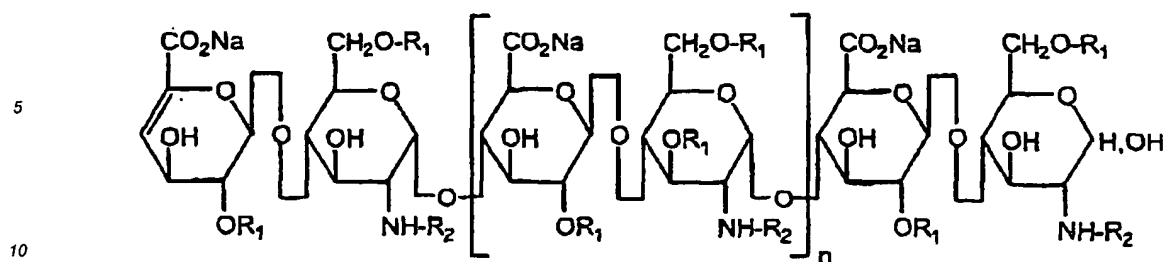
R_2SO_3Na oder $COCH_3$ ist,

umfassen, **dadurch gekennzeichnet, dass** die Zusammensetzung weniger als 10% oligosaccharidfragmente der allgemeinen Formel, in welcher n zwischen 10 und 12 liegt, 80 bis 90% Oligosaccharidfragmente der allgemeinen Formel, in welcher n zwischen 1 und 6 liegt, und weniger als 15% Oligosaccharidfragmente der allgemeinen Formel, in welcher n zwischen 7 und 9 liegt, umfasst und dass 25 bis 60% der Oligosaccharide ein Molekulargewicht von weniger als 2000 Dalton, 40 bis 75% der Oligosaccharide ein Molekulargewicht, das zwischen 2000 und 6000 Dalton liegt, und weniger als 15% der Oligosaccharide ein Molekulargewicht von größer als 6000 Dalton aufweisen, wobei das mittlere Molekulargewicht (M_w) der Zusammensetzung zwischen 2000 und 4000 Dalton liegt, wodurch die Zusammensetzung eine hohe Aktivität des Antifaktors Xa zwischen 100 und 150 I.U./mg und eine niedrige Aktivität des Antifaktors IIa von weniger als oder gleich 10 I.U./mg aufweist.

2. Verwendung der Zusammensetzung nach Anspruch 1 bei der Herstellung eines Medikaments gegen Thrombose.

Revendications

1. Composition d'héparines ayant un très bas poids moléculaire et comprenant des fragments ou oligosaccharides de formule générale:



Dans laquelle:

- 15
- n peut varier entre 1 et 12,
 R_1 est H ou SO_3Na et
 R_2 est SO_3Na ou COCH_3

20

Caractérisée par le fait que cette composition comprend moins de 10% de fragments ou oligosaccharides selon la formule générale et où n peut varier entre 10 et 12; entre 80 et 90% de fragments ou oligosaccharides selon la formule générale avec n allant entre 1 et 6; et moins de 15% de fragments ou oligosaccharides selon la formule générale pour lesquels n varie entre 7 et 9; et entre 25 et 60% de ces oligosaccharides ont un poids moléculaire inférieur à 2.000 Daltons, entre 40 et 75% de ces oligosaccharides ont un poids moléculaire allant de 2.000 à 6.000 Daltons et moins de 15% de ces oligosaccharides ont un poids moléculaire supérieur à 6.000 Daltons, le poids moléculaire moyen (M_w) de la composition varie entre 2.000 et 4.000 Daltons; raison pour laquelle cette composition présente une forte activité anti-facteur Xa entre 100 et 150 UI/mg et une faible activité anti-facteur II inférieure ou égale à 10 UI/mg.

2. Utilisation de la composition selon la revendication 1 pour la préparation d'un médicament antithrombotique.